0022-3565/01/2971-181-188$3.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

JPET 297:181–188, 2001

Vol. 297, No. 1
3240/892170
Printed in U.S.A.

Natriuretic Peptide-Induced Relaxation of Myometrium from the Pregnant Guinea Pig Is Not Mediated by Guanylate Cyclase Activation

JORGE A. CARVAJAL, KRIPAMOY AGUAN, LOREN P. THOMPSON, IRINA A. BUHIMSCHI, and CARL P. WEINER

Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Maryland Baltimore, School of Medicine, Baltimore, Maryland

Received August 21, 2000; accepted December 21, 2000 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

We tested both relaxation and cGMP generation by atrial (ANP), brain (BNP), and C-type natriuretic peptide (CNP) in oxytocin-stimulated myometrium from near-term pregnant guinea pigs to investigate the ability and mechanism of natriuretic peptides to inhibit myometrial contractility. Myometrial strips were contracted by 10^{-8} M oxytocin, and relaxation to the cumulative addition (10^{-9}–10^{-6} M) of the natriuretic peptides measured. Maximal relaxation to BNP was significantly greater than to ANP (52 versus 32% respectively; $p < 0.05$), whereas CNP failed to produce relaxation. However, the increase in cGMP produced by BNP (10^{-7} M) was significantly less than that produced by ANP (10^{-7} M) (4.5 versus 7.0 times basal; $p < 0.05$); CNP did not increase myometrial cGMP. Anantin, a competitive blocker of the guanylate cyclase A receptor, significantly reduced the increase in cGMP produced by ANP and BNP, but had no effect on relaxation induced by either peptide. Rp-8-Br-cGMP, an inhibitor of the cGMP-dependent protein kinase, did not alter BNP-induced relaxation. The atrial natriuretic peptide-fragment 4-23 amide, a natriuretic peptide clearance receptor agonist, failed to inhibit oxytocin-stimulated myometrial contraction. We conclude that natriuretic peptide induced relaxation of oxytocin-stimulated myometrium from the pregnant guinea pig is not mediated by either guanylate cyclase A or B activation, is independent of the cGMP pathway, and does not involve clearance receptor activation. Our results suggest that natriuretic peptide-induced relaxation of pregnant myometrium is mediated via a novel mechanism.

A successful pregnancy requires relaxation of the uterus during more than 95% of gestation, overcoming the inherent tendency of myometrium to contract under stretch until the appropriate time for labor. This active and highly regulated process is called myometrial quiescence. It entails not only the near absence of myometrial contractions but also its refractoriness to contractile agents. Myometrial quiescence is mediated by complex, but as yet poorly understood molecular mechanisms despite much research (Cunningham et al., 1993).

Several investigators studying rats, guinea pigs, and rabbits have documented a time-dependent increase in total myometrial cGMP content during pregnancy, which decreases shortly before the onset of labor (Weiner et al., 1994; Yallampalli et al., 1994). This temporal profile and the known smooth muscle-relaxing capability of cGMP (Lincoln et al., 1994; Vaandrager and de Jonge, 1996; Carvajal et al., 2000) lead these researchers to propose this second intracellular messenger has a central role in the maintenance of uterine quiescence, although disagreeing on the source of the cGMP (Weiner et al., 1994; Yallampalli et al., 1994).

Nitric oxide (NO) is a potent, endogenous, relaxing agent of vascular tissues, and its action is mediated predominantly by an increase in intracellular cGMP (Moncada et al., 1991). Several groups pursued the possibility that NO, by its activation of soluble guanylate cyclase, was responsible for the increase in myometrial cGMP during pregnancy and consequently was central to uterine quiescence (Natuzzi et al., 1993; Sladek et al., 1993; Bansal et al., 1997). However, we demonstrated that the increase in myometrial cGMP in the pregnant guinea pig was independent of NO production and soluble guanylate cyclase activation, and proposed that the cGMP increase was secondary to particulate guanylate cyclase activation (Weiner et al., 1994).

The natural activators of particulate guanylate cyclase receptors are members of a family of natriuretic peptides (Rosenzweig and Seidman, 1991) and their presence has been

This study was supported by grants from the National Institutes of Health: Fogarty International Center 5 F05 TW05442-02 (to J.A.C.); HL49999 (to L.P.T.); and HL49041, HD24492, and HL51735 (to C.P.W.). This work was presented in part at the 47th Annual Meeting of the Society for Gynecological Investigation in Atlanta, GA, March 2000.

ABBREVIATIONS: NO, nitric oxide; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; GC-B, guanylate cyclase B; GC-A, guanylate cyclase A; CNP, C-type natriuretic peptide; 8-Br-cGMP, 8-bromoadenosine cGMP: PKG, cGMP-dependent protein kinase; cANP, atrial natriuretic peptide des-(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})-fragment 4-23 amide; PTX, pertussis toxin; TEA, tetraethylammonium.

demonstrated in gestational tissues. Atrial natriuretic peptide (ANP) is produced by the human placenta (Graham et al., 1996). Brain natriuretic peptide (BNP) is present in amniotic fluid (Itoh et al., 1993) and produced by cultured amnion cells (Itoh et al., 1993). Furthermore, we have found high levels of BNP mRNA in human chorion taken from women at term before the onset of labor (K. Aguan, personal communication).

Natriuretic peptide receptors have been demonstrated in rat (Dos Reis et al., 1995; Vaillancourt et al., 1998), guinea pig (Aguan at al., 1998), and human myometrium (Itoh et al., 1994; Aguan et al., 1999). In the rat uterus, the protein levels of the guanylate cyclase type B receptor (GC-B) were greater than those of guanylate cyclase type A (GC-A) (Vaillancourt et al., 1998), whereas in human myometrium, it was suggested that GC-A is the dominant active receptor based on the measurement of guanylate cyclase activity (Itoh et al., 1994). Furthermore, it has been observed in all species studied to date that GC-B, but not GC-A, is down-regulated during pregnancy (Aguan et al., 1998, 1999; Vaillancourt et al., 1998).

Thus, we hypothesized that a locally produced natriuretic peptide, by activation of a particulate guanylate cyclase, is the stimulus for the increased myometrial cGMP content during pregnancy (Weiner et al., 1994). We further hypothesized that this natriuretic peptide is central to the maintenance of myometrial quiescence during pregnancy. The objective of the present study is to demonstrate the ability of natriuretic peptides to inhibit oxytocin-induced myometrial contractility during pregnancy and characterize its mechanism of action.

Materials and Methods

Animal Model. Timed pregnant, Duncan-Hartley guinea pigs were purchased from a commercial breeder (Harlan Sprague-Dawley, Indianapolis, IN). Myometrium was obtained from near-term (60 ± 2 days of gestation), anesthetized (xylazine, 4 mg/kg i.m.; ketamine hydrochloride, 100–200 mg/kg i.p.) guinea pigs respiring spontaneously. Full thickness strips opposite to the placental implantation site were excised, including decidua but excluding fetal membranes. The myometrial strips were placed in Krebs' buffer and stored on wet ice until used within 1 h for isometric tension recording. The Committee on Animal Care at the University of Maryland Baltimore approved the protocol.

The guinea pig was selected as a model for several reasons: 1) its hemomonochorial placentation, which is the most similar to human placenta among all nonprimate mammals (Pijnenborg et al., 1981); 2) the similarity to humans of its sex hormone profile during the estrus cycle and pregnancy; and 3) the relatively long and stable gestational period (65 ± 2 days from copulation), making easy comparisons between different gestational ages.

Isometric Tension Studies. Longitudinal strips (~10 mm in length × 2 mm in width) of full thickness myometrium were placed in organ chambers and attached to a force transducer (Grass Instruments, Quincy, MA) for isometric tension recording. The chambers contained Krebs' buffer composed of 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 11.1 mM D-glucose, 0.016 mM EDTA, 2.2 mM $CaCl_2$, 15.8 mM $NaHCO_3$, pH = 7.35 to 7.45, maintained at 37°C, and continuously bubbled with 95% O_2, 5% CO_2. The myometrial strips were equilibrated under 1-g passive tension until a stable baseline, typically around 0.5-g basal tension (range 0.4–0.6 g) was achieved (approximately 30 min). The bath buffer solution was changed every 5 min during equilibration. After the stabilization period, contractions were produced by a submaximal concentration of oxytocin (10^{-8} M), which approximated the EC_{50} obtained from concentration-response curves previously determined. The experiment was begun after a regular pattern of contractions was achieved, typically 15 to 20 min after the addition of the oxytocin.

The spontaneous contractile activity of isolated guinea pig myometrial strips was characterized by irregular contractions of long duration (approximately 3 min for each contraction), high amplitude and variable frequency (0–4/10 min). Oxytocin initially produced a tetanic contraction lasting 3 to 5 min. Tetany was followed by regular contractions of short duration (30–60 s), low amplitude compared with the spontaneous contractions, with a frequency of 10 to 15 in 10 min. This pattern of activity continued with little variation for about 30 min. Thereafter, the contractile activity declined somewhat by 60 min after initiating the experiment to ≈80% of the oxytocin-stimulated basal activity (p = N.S.). In preliminary studies, we determined that 10^{-8} M oxytocin was the optimal concentration to produce this regular pattern of contractions.

Contractions were recorded and analyzed using PowerLab/800 hardware and Chart v3.4 software (AD Instruments, Mountain View, CA). To quantify contractile activity, the integral area under the curve over 5-min intervals was measured and normalized for the cross-sectional area of the strip. The cross-sectional area was calculated as W/(L × D), where W is weight (g), L is length (cm), and D (density) is 1.05 g/cm^3. Basal activity was designated as the stabilized contractile response to oxytocin during the 5-min period before the addition of peptides. The effect of natriuretic peptides on myometrial contractility was measured as the activity during the 5-min period after their addition to the organ bath. The difference between the basal activity and that after the addition of natriuretic peptide was calculated and expressed as a percentage of the basal activity, and identified as the percentage of relaxation.

Effect of Natriuretic Peptides on Oxytocin-Induced Contractility. The effect of natriuretic peptides on myometrial contractility was measured by generating concentration-response curves to the cumulative addition of each peptide. Contractility was induced by oxytocin (10^{-8} M) and relaxation to ANP, BNP, and C-type natriuretic peptide (CNP) determined over a concentration range of 10^{-9} to 10^{-6} M (final concentration in the organ bath). The relaxation response was measured during the 5-min interval after addition of the natriuretic peptide and compared with the basal contractile activity. The 5-min interval was selected for analysis of the dose response because the temporal profile of natriuretic peptide-induced relaxation showed an immediate effect of the peptides that began to decrease after 5 min.

The response to a single concentration of each natriuretic peptide (10^{-7} M) was used to determine the temporal course of relaxation for each natriuretic peptide by recording myometrial contractility for 30 min after peptide addition. The effect was measured in 5-min intervals, represented in the text and figures by their mid-time point (e.g., 2.5 min, 7.5 min, etc.)

Effect of 8-Br-cGMP on Oxytocin-Stimulated Myometrial Contractility. To test the ability of cGMP to modulate oxytocin-induced contractile activity of myometrium from pregnant guinea pig, a concentration-response curve was prepared to 8-Br-cGMP, a membrane-permeable cGMP analog.

After the presence of regular and stable contractions was documented, the test drug was added directly to the organ chamber in 0.5 log-unit increments (in volumes of 20–40 μl as appropriate). A new dose was added every 10 min. Vehicle controls were run in parallel. The amount of drug used was expressed as its final concentration in the organ bath. The effect of 8-Br-cGMP was expressed as the contractile activity during the 10-min interval after the addition of the drug, and compared with its basal activity.

Mechanism of Natriuretic Peptide-Induced Relaxation. To determine whether natriuretic peptide-induced relaxation occurred by activation of particulate GC-A, myometrial strips were preincubated for 5 min with anantin (10^{-6} M), a competitive antagonist of the GC-A receptor, before the addition of a single dose of natriuretic

peptide. Because the single-dose studies revealed that the relaxation induced by natriuretic peptides was maximal during the first 5 min, we selected this interval to compare the effect of anantin alone, and the effect of anantin on natriuretic peptide-induced relaxation.

To determine whether natriuretic peptides relaxed myometrium by increasing cGMP, the cGMP pathway was blocked by a 30-min preincubation with 30 μM Rp-8-Br-cGMP, a membrane-permeable cGMP analog that specifically inhibits cGMP-dependent protein kinase (PKG), before the addition of a single dose of BNP. This incubation time and concentration had previously been shown in vascular tissues to inhibit PKG activation and relaxation induced by the cGMP pathway (Dhanakoti et al., 2000). We, too, tested whether this incubation period and drug concentration were adequate to inhibit PKG by measuring the PKG activity in three different experiments performed in duplicate. PKG activity was determined as described by measuring the incorporation of $^{32}P_i$ into a PKG-specific substrate (Diwan et al., 1994; Patel and Diamond, 1997). Basal myometrial PKG activity was 16% of the maximal activity induced by 5 μM cGMP. Five minutes after the addition of a single dose (10^{-7} M) of BNP, PKG activity increased to 40% of maximum. This effect of BNP was almost completely blocked by the 30-min incubation with 30 μM Rp-8-Br-cGMP; PKG activity was only 10% of maximum under this condition.

Some of the biological actions of natriuretic peptides are mediated by activation of the natriuretic peptide clearance receptor (Anand-Srivastava and Trachte, 1993; Hempel et al., 1998). To determine whether clearance receptor activation is part of the biological action of the natriuretic peptides on myometrial contractility, the effect of a single dose of atrial natriuretic peptide des-(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})-fragment 4-23 amide (cANP), a specific agonist of the natriuretic peptide clearance receptor, on oxytocin-induced contractility was tested. It has been reported that natriuretic peptide action via the clearance receptor involves a heterotrimeric G protein-coupled mechanism (Anand-Srivastava and Trachte, 1993; Murthy and Makhlouf, 1999) and results in an increase in K^+ outward conductance (Anand-Srivastava and Trachte, 1993; Kanwal and Trachte, 1994). To determine whether the mechanism of natriuretic peptide relaxation involved a pertussis toxin (PTX)-sensitive G protein, myometrial strips were preincubated for 3 h with PTX (5 μg/ml), a specific blocker of heterotrimeric G protein α_i-subunit, before the addition of BNP to oxytocin-stimulated myometrium. This concentration and incubation time had been shown to effectively block PTX-sensitive G protein in porcine myometrial strips (Kitazawa et al., 2000). The effect of 2 mM tetraethylammonium (TEA), a nonspecific K^+ channel blocker, on BNP-induced relaxation was studied by adding the drug to the organ bath 5 min before the addition of BNP. At this concentration and incubation time, TEA effectively blocks myometrial K^+ channels (Perez et al., 1993; Khan et al., 1997).

cGMP Measurement by Radioimmunoassay. To determine the effect of natriuretic peptides on particulate guanylate cyclase activation, we measured the cGMP content of guinea pig myometrial samples both under basal condition and after the stimulus of a single dose (10^{-7} M) of ANP, BNP, and CNP, either in the presence or absence of anantin (10^{-6} M). To correlate the effect of natriuretic peptides on cGMP levels with their effect on contractility, three additional experiments were performed (in duplicate) in the presence of 10^{-8} M oxytocin, the same concentration used to induce contraction of the myometrial strips. To determine the temporal profile of natriuretic peptide-induced myometrial cGMP content, cGMP was measured in duplicate tissue samples every 5 min beginning 2.5 min after the addition of the peptide (up to 27.5 min total).

cGMP was measured as previously described (Weiner et al., 1994). At the appropriate time, myometrial samples (about 5 mm^2) were placed directly into boiling 50 mM Tris with 4 mM EDTA at a pH of 7.5 for 10 min. The tissue was then minced, homogenized, centrifuged at 14,000g (−4°C) for 20 min, and the supernatant snap frozen until assayed for cGMP content. The total protein content of the pellet was measured spectrophotometrically using bicinchoninic acid method (BCA kit; Pierce, Rockford, IL). The frozen supernatants were thawed and diluted (1:75) in 25 mM Tris/4 mM EDTA, pH 7.5. cGMP content was measured by radioimmunoassay using a commercially available kit (Amersham, Piscataway, NJ). All cGMP measurements were corrected for total protein content in the sample. Duplicate values were averaged and the measurements expressed as picomoles per milligram protein. To represent the changes in cGMP content, we compared the cGMP levels induced by natriuretic peptides with their basal level and expressed the results as times basal. Intra- and interassay variations were each less than 5%.

Drugs and Solutions. Oxytocin, human ANP, BNP, and CNP, anantin, Rp-8-Br-cGMP, cANP, PTX, and TEA were obtained from Sigma Chemical Co. (St. Louis, MO). All drugs were dissolved in double deionized water.

Statistical Analysis. All data sets (mean ± standard error of the mean) were subject to a test of normalcy (Shapiro-Wilk test) and parametric or nonparametric tests performed where appropriate. Statistical comparisons between two groups were performed using Student's t test. For multiple group comparisons, either one-way ANOVA followed by post hoc Student-Newman-Keuls (parametric) or Kruskall-Wallis one-way ANOVA on ranks followed by Dunnett's multiple comparison test (nonparametric) was used. A two-tailed $p < 0.05$ was considered indicative of statistical significance.

Results

Effect of Natriuretic Peptides on Oxytocin-Induced Contractility. Both ANP and BNP, but not CNP, produced dose-dependent relaxation of oxytocin-induced myometrial contractility (Fig. 1). The effect of BNP was significantly greater than ANP ($p < 0.05$), and the relaxation curve to ANP did not lie to the left of the relaxation curve to BNP, surprising results considering that ANP, compared with BNP, has greater potency and affinity for the GC-A receptor. The threshold concentration for both BNP and ANP was 10^{-8} M; relaxation induced at 10^{-6} M (the maximal dose used), was 52 and 32% respectively ($p < 0.05$).

Temporal Course of Natriuretic Peptide-Induced Relaxation. Figure 2 illustrates the effect of a single concentration (10^{-7} M) of ANP and BNP on oxytocin-induced contractility. During the first 5 min, the magnitude of the

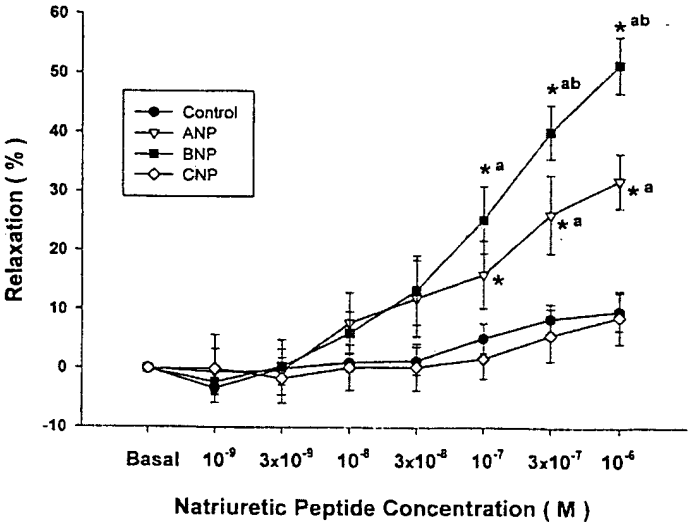

Fig. 1. Effect of natriuretic peptides on oxytocin-induced contractility of pregnant guinea pig myometrium. Values are mean ± S.E.M. from five experiments in duplicate. $^*p < 0.05$ versus basal; $^ap < 0.05$ versus control; $^bp < 0.05$ versus ANP.

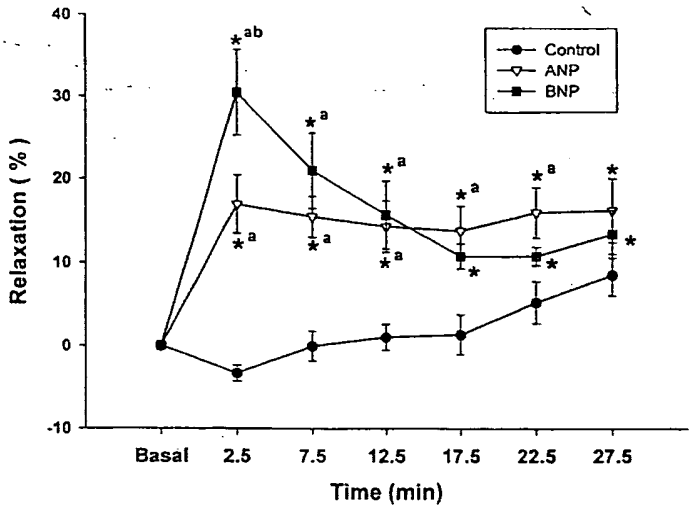

Fig. 2. Temporal course of a single-dose natriuretic peptide (10^{-7} M)-induced relaxation of oxytocin-induced contractility. Times in minutes are 5-min intervals represented by the mid-time point. Values are mean ± S.E.M. from five experiments in duplicate. $^{*}p < 0.05$ versus basal; $^{a}p < 0.05$ versus control; $^{b}p < 0.05$ versus ANP.

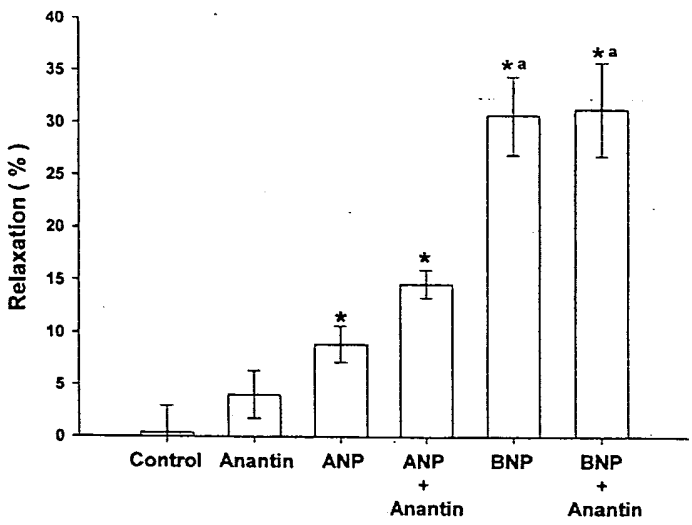

Fig. 4. Effect of anantin (10^{-6} M) on single-dose natriuretic peptide (10^{-7} M)-induced relaxation of oxytocin-induced contractility. Anantin was added to the bath 5 min before natriuretic peptide. Contractility was measured in the 5-min interval after drug addition. Values are mean ± S.E.M. from five experiments in duplicate. $^{*}p < 0.05$ versus control; $^{a}p < 0.05$ versus ANP.

relaxation induced by BNP was greater than ANP (31 versus 12% relaxation, respectively; $p < 0.05$).

The contractile activity of the control strips decreased slightly over time, but was never significantly different from the basal level. The relaxation induced by BNP, but not ANP, decreased significantly over time, but relaxation to both peptides remained significantly greater than the basal level throughout the 30-min study period ($p < 0.05$, Fig. 2) and compared with their temporal control at each time point up to 12.5 min for BNP and 22.5 min for ANP ($p < 0.05$, Fig. 2).

Effect of 8-Br-cGMP on Oxytocin-Stimulated Myometrial Contractility. 8-Br-cGMP produced a dose-dependent decrease in oxytocin-stimulated contractile activity in myometrium of pregnant guinea pig. Relaxation was apparent at 10^{-5} M, ultimately reaching 63% relaxation from baseline at 3×10^{-4} M (the maximal concentration used) as illustrated in Fig. 3.

Effect of Anantin on Natriuretic Peptide-Induced Relaxation. Figure 4 illustrates the effect of anantin (10^{-6} M) on the natriuretic peptide-induced relaxation during the first 5-min period. There was no effect of anantin alone on oxytocin-induced contractility. Alone, both ANP and BNP relaxed oxytocin-stimulated myometrium by 9 and 31%, respectively ($p < 0.05$). There was no effect of anantin on the relaxation induced by a single concentration (10^{-7} M) of either ANP or BNP.

Effect of Rp-8-Br-cGMP on BNP-Induced Relaxation. The effect of Rp-8-Br-cGMP on BNP-induced relaxation of oxytocin-stimulated myometrium is illustrated in Fig. 5. Under control conditions, a single dose of 10^{-7} M BNP produced a 30% relaxation. The addition of 30 μM Rp-8-Br-cGMP (30-min preincubation) had no effect on BNP-induced relaxation (32% relaxation, Fig. 5).

Effect of cANP on Oxytocin-Induced Contractility. The atrial natriuretic peptide-fragment cANP, a specific ag-

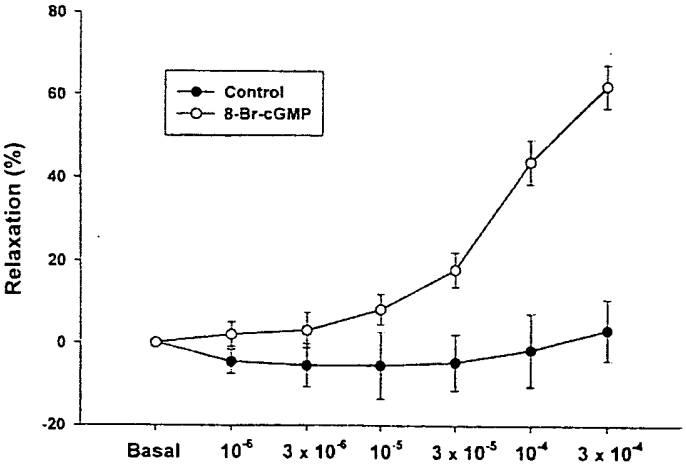

Fig. 3. Effect of 8-Br-cGMP on oxytocin-stimulated contractility of pregnant guinea pig myometrium. Values are mean ± S.E. from five experiments in duplicate.

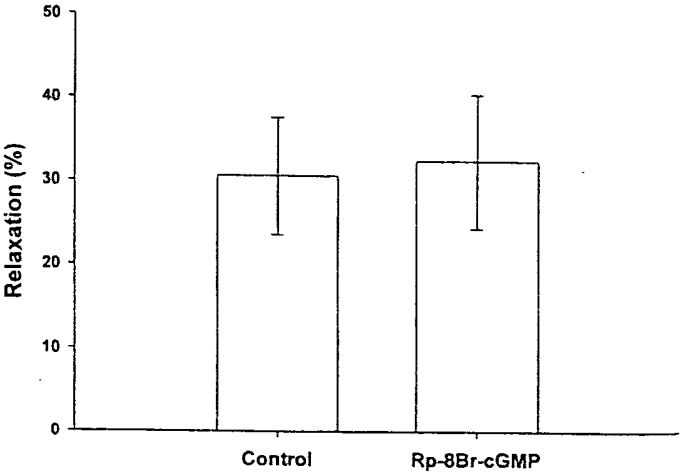

Fig. 5. Effect of Rp-8-Br-cGMP (30 μM) on single-dose BNP (10^{-7} M)-induced relaxation of oxytocin-induced contractility. Strips were preincubated with Rp-8-Br-cGMP by 30 min. Contractility was measured in the 5-min interval after BNP addition. Values are mean ± S.E.M. from five experiments in duplicate.

onist of the natriuretic peptide clearance receptor, had no significant effect on oxytocin-induced myometrial contractions at either 10^{-7} or 10^{-6} M (Fig. 6).

Effect of PTX and TEA on BNP-Induced Relaxation. The effects of preincubation with either PTX or TEA on BNP-induced relaxation are illustrated in Fig. 7. Under control conditions, BNP at 10^{-7} M produced 26% relaxation; neither PTX (5 μg/ml) nor TEA (2 mM) had any effect on BNP-induced relaxation of oxytocin-stimulated myometrial contractions (Fig. 7).

Natriuretic Peptide-Induced Myometrial cGMP Generation. Both ANP and BNP, but not CNP, increased myometrial cGMP content (Fig. 8). Myometrial cGMP content under basal conditions was 7.41 ± 1.6 pmol/mg of protein (average ± S.E.). After the addition of 10^{-7} M ANP or BNP, cGMP rose to 45.14 ± 9.5 and 28.87 ± 4.58 pmol/mg of protein, respectively ($p < 0.05$).

As illustrated in Fig. 8, a single concentration (10^{-7} M) of ANP, compared with BNP, generated a larger increase in cGMP at 2.5 min (7- versus 4.5-fold increase above the basal level, respectively; $p < 0.05$). This finding is consistent with the reported higher GC-A receptor affinity of ANP compared with BNP. The results of this experiment were not affected by the presence of 10^{-8} M oxytocin. In this situation, basal levels of cGMP were increased 6.5-fold by 10^{-7} M ANP and 4.5-fold by 10^{-7} M BNP ($p < 0.05$).

The temporal course of the cGMP increase (Fig. 8) paralleled the relaxation profile produced by both ANP and BNP (Fig. 2). cGMP content decreased over time, but remained significantly elevated over the basal level at the end of the experiment ($p < 0.05$).

Effect of Anantin on Natriuretic Peptide-Induced cGMP Generation. Figure 9 illustrates the effect of anantin (10^{-6} M) on myometrial cGMP content 2.5 min after a single concentration (10^{-7} M) of either ANP or BNP. Anantin alone had no effect on cGMP content (6.57 ± 2.0 pmol/mg of protein), compared with the basal myometrial cGMP level of 5.26 ± 1.1 pmol/mg of protein.

Alone, ANP increased cGMP to 34.95 ± 6.7 pmol/mg. The effect of ANP was significantly reduced by anantin; cGMP rose only to 21.77 ± 4.1 pmol/mg ($p < 0.05$). Similarly, BNP increased cGMP to 22.38 ± 3.4, but only to 14.17 ± 2.64 in the presence of anantin ($p < 0.05$). Therefore, as shown in Fig. 9, anantin significantly inhibited about 40% the cGMP rise stimulated by both ANP and BNP. The addition of oxytocin to the tissue bath did not alter this response; ANP compared with BNP produced a larger increase in cGMP, and the rise in cGMP was significantly inhibited by 10^{-6} M anantin. Under this condition, ANP increase myometrial cGMP 6.5- and 4.5-fold either in the absence or presence of anantin, respectively. And BNP increase myometrial cGMP (in the presence of oxytocin) 4.5- and 2-fold (absence versus presence of anantin, respectively).

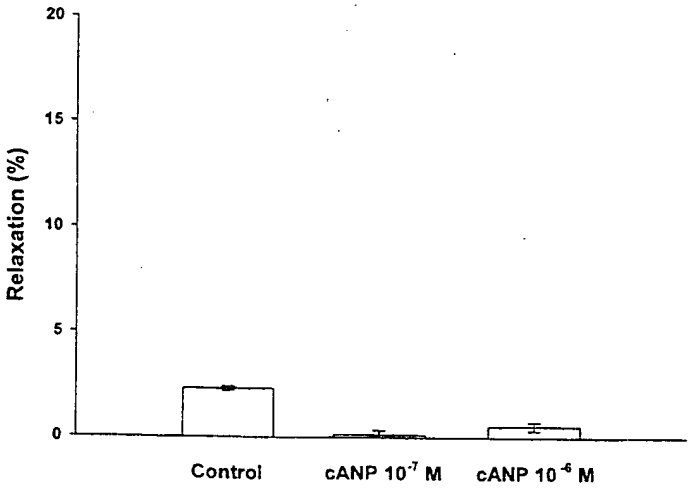

Fig. 6. Effect of a single dose of cANP on oxytocin-induced contractility of pregnant guinea pig myometrium. Values are mean ± S.E.M. from five experiments in duplicate.

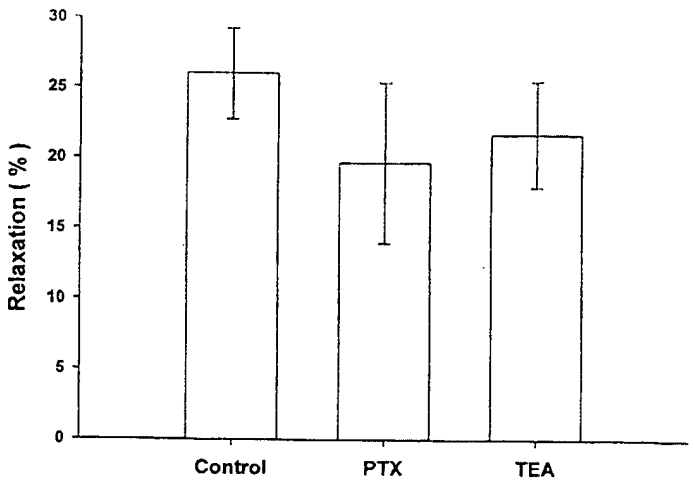

Fig. 7. Effect of PTX (5 μg/ml) and TEA (2 mM) on single-dose BNP (10^{-7} M)-induced relaxation of oxytocin-induced contractility. Strips were preincubated with PTX by 3 h. TEA was added to the bath 5 min before BNP. Contractility was measured in the 5-min interval after BNP addition. Values are mean ± S.E.M. from five experiments in duplicate.

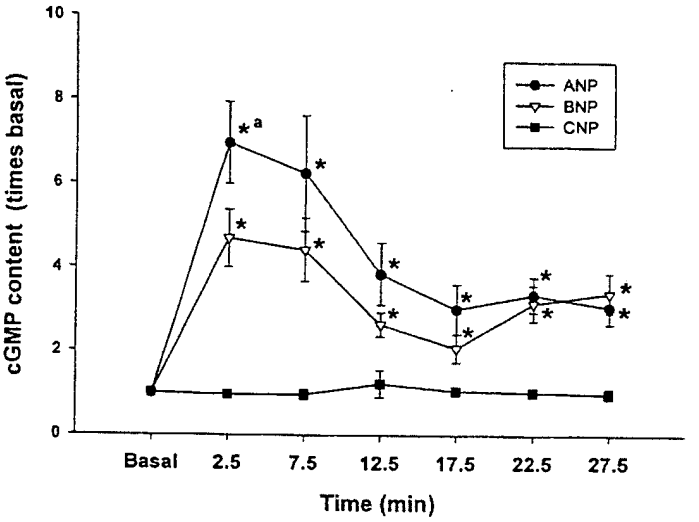

Fig. 8. Temporal course of myometrial cGMP generation induced by a single dose (10^{-7} M) of natriuretic peptides. cGMP was measured at basal time and every 5 min starting at 2.5 min after natriuretic peptide addition. Values are mean ± S.E.M. from five experiments in duplicate. $^{*}p < 0.05$ versus basal; $^{a}p < 0.05$ versus BNP.

Discussion

We tested the ability of natriuretic peptides to decrease oxytocin-induced myometrial contractility. Our studies reveal that ANP and BNP, but not CNP, inhibit oxytocin-

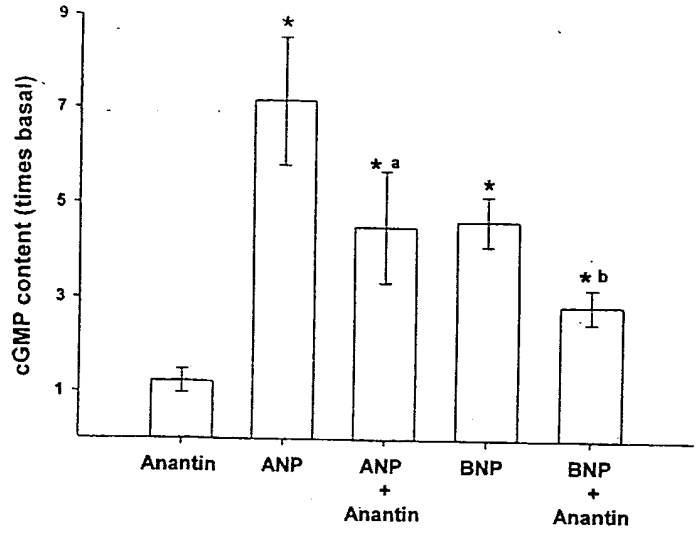

Fig. 9. Effect of anantin (10^{-7} M) on myometrial cGMP levels induced by a single dose (10^{-7} M) of natriuretic peptides. Anantin was added to the bath 5 min before natriuretic peptide. cGMP was measured 2.5 min after natriuretic peptide addition. Values are mean ± S.E.M. from five experiments in duplicate. $^{*}p < 0.05$ versus basal; $^{a}p < 0.05$ versus ANP; $^{b}p < 0.05$ versus BNP.

induced contractions of myometrium obtained from near-term pregnant guinea pigs. Furthermore, although BNP produced greater relaxation than ANP, ANP stimulated a greater increase in cGMP. Anantin had no effect on natriuretic peptide-induced relaxation, but inhibits the increase in cGMP stimulated by both ANP and BNP. The results of these experiments suggest that natriuretic peptide-induced relaxation of myometrium from the pregnant guinea pig is not mediated by receptor guanylate cyclase activation.

Six isoforms of the receptors for natriuretic peptides with guanylate cyclase activity have been identified, in addition to the nonguanylate cyclase clearance receptor (Foster et al., 1999). The type A (GC-A) and type B (GC-B) receptors have been demonstrated in rat (Dos Reis et al., 1995: Vaillancourt et al., 1998), guinea pig (Aguan et al., 1998), and human myometrium (Itoh et al., 1994; Aguan et al., 1999). The binding affinity and the order of potency for GC-A activation is ANP ≥ BNP >>> CNP, whereas for GC-B it is CNP ≫ ANP ≥ BNP (Suga et al., 1992). The results of our experiments on natriuretic peptide-induced myometrial relaxation are inconsistent with the above-described receptor affinity because of the absent left shift in the relaxation response to ANP compared with BNP.

In the present study, BNP was a more effective inhibitor of oxytocin-induced myometrial contractions than ANP; CNP had no effect on contractility. Since CNP does not induce relaxation of guinea pig myometrium, we conclude that GC-B activation is not involved in mediating the studied response. Our relaxation-response curve to natriuretic peptides did not show a larger affinity of ANP compared with BNP, and anantin, a specific, competitive blocker of GC-A (Weber et al., 1991), had no effect on the relaxation induced by both peptides. This supports our conclusion that BNP-induced relaxation is not mediated by GC-A activation. Based on these results, we suggest that BNP induces relaxation by a mechanism that does not involve activation of either GC-A or GC-B.

In contrast to relaxation, natriuretic peptides increase myometrial cGMP by GC-A activation. The functional presence of GC-A in myometrium obtained from pregnant guinea pigs and its higher affinity for ANP were demonstrated by the significantly larger increase in cGMP content induced by ANP compared with BNP. Furthermore, anantin significantly reduced the ANP- and BNP-mediated increase in myometrial cGMP content, suggesting that both peptides activate GC-A to increase cGMP. Additionally, CNP did not produce any significant change in myometrial cGMP content, suggesting that GC-B activation is not related to cGMP production in the pregnant guinea pig.

Although the time course of the natriuretic peptide-induced cGMP increase paralleled the relaxing effect of the peptides on myometrial contractility, anantin had no effect on natriuretic peptide-induced relaxation, while inhibiting the peptide-stimulated increase in cGMP. Additionally, inhibition of cGMP-dependent protein kinase with Rp-8-Br-cGMP also did not alter the relaxation response to BNP. These results suggest that the cGMP pathway does not mediate the relaxation induced by ANP and BNP. The conclusion that neither GC-A activation nor the resulting cGMP increase mediates BNP-induced relaxation is supported by the observation that whereas BNP-induced relaxation was greater than that induced by ANP, the stimulated increase in cGMP content was less. Therefore, we conclude that the relaxation of pregnant guinea pig myometrium to natriuretic peptides is mediated by activation of a receptor distinct from GC-A and GC-B, which lacks guanylate cyclase activity.

This conclusion is central to the support of our hypothesis of a role for natriuretic peptides in the maintenance of uterine quiescence, because it has been reported that the cGMP-PKG pathway was uninvolved in the regulation of myometrial contractility during pregnancy (Word and Cornwell, 1998). These authors found that cGMP analogs produced only modest relaxation of myometrial preparations of rat uterus and concluded that pregnant rat myometrium was insensitive to cGMP-induced relaxation (Word and Cornwell, 1998). This interpretation is consistent with our finding that cGMP analogs produced significant relaxation of oxytocin-induced myometrial contractility only at high concentrations. Furthermore, myometrial insensitivity to cGMP was explained because of a pregnancy-induced decrease PKG expression and function (Word and Cornwell, 1998). This line of evidence could be used to argue against a role for natriuretic peptides in the maintenance of uterine quiescence during pregnancy if their action was PKG mediated. However, the interpretation of our results refute this argument since we document that natriuretic peptide-induced relaxation occurs via a pathway that does not involve either cGMP or PKG activation.

There is a minor concern regarding the experiments using Rp-8-Br-cGMP to inhibit PKG activity. The effectiveness of this cGMP analog in inhibiting the activity of the cGMP-dependent protein kinase is supported by previous work (Butt et al., 1994; Patel and Diamond, 1997), and our experiments do demonstrate a decrease in PKG activity after incubation with 30 μM Rp-8-Br-cGMP. However, we cannot exclude the chance that the inhibition detected is explained by an effect of the drug on PKG during the PKG assay after tissue homogenization rather than its effect on the intact tissue. This possibility, however, does not argue against our

conclusion that natriuretic peptide-induced relaxation does not occur trough the cGMP-PKG pathway, since the lines of evidence presented supporting our conclusion are multiple and do not only rely solely on the use of this cGMP analog.

The observation that cGMP analogs such as 8-Br-cGMP require a high concentration to produce relaxation in the myometrial strips should be carefully considered. It is difficult to interpret whether this is due to poor tissue permeability, or the minimal role of cGMP generated by the myometrium in relaxation. Furthermore, this may also indicate that although exogenous administration of cGMP causes relaxation, agonist-stimulation of endogenous cGMP is either not sufficient or is compartmentalized in the cell and is not a significant second messenger.

It has been reported that natriuretic peptides can have biological actions not mediated by guanylate cyclase receptor activation and cGMP production, but by a signaling activity via the natriuretic peptide clearance receptor (Johnson et al., 1991; Hempel et al., 1998). This signaling pathway does not lead to cGMP generation (Drewett et al., 1992), and involves a pertussis toxin-sensitive G protein (Anand-Srivastava et al., 1996; Murthy et al., 1998; Murthy and Makhlouf, 1999). Activation of the clearance receptor inhibits adenylate cyclase (Drewett et al., 1992), increases phospholipase C activity (Anand-Srivastava and Trachte, 1993), and increases K^+ outward conductance (Anand-Srivastava and Trachte, 1993; Kanwal and Trachte, 1994). Although a relationship between clearance receptor activation and myometrial contraction/relaxation has not been shown, the presence of the natriuretic peptide clearance receptor mRNA has been identified in the rat myometrium (Vaillancourt et al., 1998). In the present study, we report that cANP, a specific agonist of the clearance receptor (Maack et al., 1987), does not inhibit oxytocin-induced contractility. Although the presence of the clearance receptor in the myometrium of the pregnant guinea pig has yet to be demonstrated, our studies do not support a role for it (if present) in the inhibition of oxytocin-induced contraction by natriuretic peptides. In addition, neither PTX (a trimeric G protein inhibitor) nor TEA (a K^+ channel blocker) interfered with BNP-induced relaxation of oxytocin-induced contractions, further supporting our conclusion that natriuretic peptides do not activate the clearance receptor to cause relaxation.

In conclusion, both BNP and ANP, but not CNP, inhibit oxytocin-induced contractions of the pregnant guinea pig myometrium. We propose that natriuretic peptide-induced relaxation is mediated by activation of a previously undescribed receptor molecule that lacks guanylate cyclase activity, and therefore does not involve the cGMP pathway. Our findings suggest that BNP has a greater efficacy in activating this receptor molecule than either ANP or CNP. We suggest that this pathway of relaxation is via a novel mechanism, independent of GC-A or CG-B activation, cGMP generation, or clearance receptor activation. Further studies are necessary to elucidate the molecular mechanism of natriuretic peptide-induced myometrial relaxation of the pregnant guinea pig myometrium.

References

Aguan K, Kramer W, Carvajal J, Thompson L and Weiner CP (1999) Expression analysis of natriuretic peptides (ANP, BNP and CNP) and their receptors (GCA and GCB) mRNAs in nonpregnant vs. pregnant human uterine tissues. *J Soc Gynecol Invest* 6:A157.

Aguan K, Mozumdar J, Thompson LP and Weiner CP (1998) Pregnancy selectively downregulates GCB receptor but not GCA receptor mRNA in guinea pig myometrium. *J Soc Gynecol Invest* 5:A186.

Anand-Srivastava MB, Sehl PD and Lowe DG (1996) Cytoplasmic domain of natriuretic peptide receptor-C inhibits adenylyl cyclase. Involvement of a pertussis toxin-sensitive G protein. *J Biol Chem* 271:19324–19329.

Anand-Srivastava MB and Trachte GJ (1993) Atrial natriuretic factor receptors and signal transduction mechanisms. *Pharmacol Rev* 45:455–497.

Bansal RK, Goldsmith PC, He Y, Zaloudek CJ, Ecker JL and Riemer RK (1997) A decline in myometrial nitric oxide synthase expression is associated with labor and delivery. *J Clin Invest* 99:2502–2508.

Butt E, Eigenthaler M and Genieser HG (1994) (Rp)-8-pCPT-cGMPS, a novel cGMP-dependent protein kinase inhibitor. *Eur J Pharmacol* 269:265–268.

Carvajal JA, Germain AM, Huidobro-Toro JP and Weiner CP (2000) Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol* 184:409–420.

Cunningham FG, MacDonald PC, Gant NF, Leveno KJ and Gilstrop LC III (1993) Parturition: Biomolecular and physiologic processes, in *Williams Obstetrics*, 19th ed. (Cunningham FG, MacDonald PC, Gant NF, Leveno KJ and Gilstrop LC III eds) pp 297–361, Appleton & Lange, Norwalk, CT.

Dhanakoti SN, Gao Y, Nguyen MQ and Raj JU (2000) Involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP. *J Appl Physiol* 88:1637–1642.

Diwan AH, Thompson WJ, Lee AK and Strada SJ (1994) Cyclic GMP-dependent protein kinase activity in rat pulmonary microvascular endothelial cells. *Biochem Biophys Res Commun* 202:728–735.

Dos Reis AM, Fujio N, Dam TV, Mukaddam-Daher S, Jankowski M, Tremblay J and Gutkowska J (1995) Characterization and distribution of natriuretic peptide receptors in the rat uterus. *Endocrinology* 136:4247–4253.

Drewett JG, Ziegler RJ and Trachte GJ (1992) Neuromodulatory effects of atrial natriuretic peptides correlate with an inhibition of adenylate cyclase but not an activation of guanylate cyclase. *J Pharmacol Exp Ther* 260:689–696.

Foster DC, Wedel BJ, Robinson SW and Garbers DL (1999) Mechanisms of regulation and functions of guanylyl cyclases. *Rev Physiol Biochem Pharmacol* 135:1–39.

Graham CH, Watson JD, Blumenfeld AJ and Pang SC (1996) Expression of atrial natriuretic peptide by third-trimester placental cytotrophoblasts in women. *Biol Reprod* 54:834–840.

Hempel A, Noll T, Bach C, Piper HM, Willenbrock R, Hohnel K, Haller H and Luft FC (1998) Atrial natriuretic peptide clearance receptor participates in modulating endothelial permeability. *Am J Physiol* 275:H1818–H825.

Itoh H, Sagawa N, Hasegawa M, Nanno H, Kobayashi F, Ihara Y, Mori T, Komatsu Y, Suga S, Yoshimasa T, et al. (1994) Expression of biologically active receptors for natriuretic peptides in the human uterus during pregnancy. *Biochem Biophys Res Commun* 203:602–607.

Itoh H, Sagawa N, Hasegawa M, Okagaki A, Inamori K, Ihara Y, Mori T, Ogawa Y, Suga S, Mukoyama M, et al. (1993) Brain natriuretic peptide is present in the human amniotic fluid and is secreted from amnion cells. *J Clin Endocrinol Metab* 76:907–911.

Johnson BG, Trachte GJ and Drewett JG (1991) Neuromodulatory effect of the atrial natriuretic factor clearance receptor binding peptide, cANF(4-23)-NH2 in rabbit isolated vasa deferentia. *J Pharmacol Exp Ther* 257:720–726.

Kanwal S and Trachte GJ (1994) Potassium channel inhibitors attenuate neuromodulatory effects of atrial natriuretic factor in the rabbit isolated vas deferens. *J Pharmacol Exp Ther* 268:117–123.

Khan RN, Smith SK, Morrison JJ and Ashford ML (1997) Ca2+ dependence and pharmacology of large-conductance K+ channels in nonlabor and labor human uterine myocytes. *Am J Physiol* 273:C1721–C1731.

Kitazawa T, Maezono Y and Taneike T (2000) The mechanisms of alpha(2)-adrenoceptor agonist-induced contraction in longitudinal muscle of the porcine uterus. *Eur J Pharmacol* 390:185–195.

Lincoln TM, Komalavilas P and Cornwell TL (1994) Pleiotropic regulation of vascular smooth muscle tone by cyclic GMP-dependent protein kinase. *Hypertension* 23:1141–1147.

Maack T, Suzuki M, Almeida FA, Nussenzveig D, Scarborough RM, McEnroe GA and Lewicki JA (1987) Physiological role of silent receptors of atrial natriuretic factor. *Science (Wash DC)* 238:675–678.

Moncada S, Palmer RM and Higgs EA (1991) Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43:109–142.

Murthy KS and Makhlouf GM (1999) Identification of the G protein-activating domain of the natriuretic peptide clearance receptor (NPR-C). *J Biol Chem* 274: 17587–17592.

Murthy KS, Teng B, Jin J and Makhlouf GM (1998) G protein-dependent activation of smooth muscle eNOS via natriuretic peptide clearance receptor [see comments]. *Am J Physiol* 275:C1409–C1416.

Natuzzi ES, Ursell PC, Harrison M, Buscher C and Riemer RK (1993) Nitric oxide synthase activity in the pregnant uterus decreases at parturition. *Biochem Biophys Res Commun* 194:1–8.

Patel AI and Diamond J (1997) Activation of guanosine 3',5'-cyclic monophosphate (cGMP)-dependent protein kinase in rabbit aorta by nitroglycerin and sodium nitroprusside. *J Pharmacol Exp Ther* 283:885–893.

Perez GJ, Toro L, Erulkar SD and Stefani E (1993) Characterization of large-conductance, calcium-activated potassium channels from human myometrium. *Am J Obstet Gynecol* 168:652–660.

Pijnenborg R, Robertson WB, Brosens I and Dixon G (1981) Review article: Trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. *Placenta* 2:71–91.

Rosenzweig A and Seidman CE (1991) Atrial natriuretic factor and related peptide hormones. *Annu Rev Biochem* 60:229–255.

Sladek SM, Regenstein AC, Lykins D and Roberts JM (1993) Nitric oxide synthase

activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am J Obstet Gynecol* 169:1285–1291.

Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K, et al. (1992) Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* 130:229–239.

Vaandrager AB and de Jonge HR (1996) Signalling by cGMP-dependent protein kinases. *Mol Cell Biochem* 157:23–30.

Vaillancourt P, Omer S, Deng XF, Mulay S and Varma DR (1998) Differential effects of rat pregnancy on uterine and lung atrial natriuretic factor receptors. *Am J Physiol* 274:E52–E56.

Weber W, Fischli W, Hochuli E, Kupfer E and Weibel EK (1991) Anantin—a peptide antagonist of the atrial natriuretic factor (ANF). I. Producing organism, fermentation, isolation and biological activity. *J Antibiot* 44:164–171.

Weiner CP, Knowles RG, Nelson SE and Stegink LD (1994) Pregnancy increases guanosine 3',5'-monophosphate in the myometrium independent of nitric oxide synthesis. *Endocrinology* 135:2473–2478.

Word RA and Cornwell TL (1998) Regulation of cGMP-induced relaxation and cGMP-dependent protein kinase in rat myometrium during pregnancy. *Am J Physiol* 274:C748–C756.

Yallampalli C, Izumi H, Byam-Smith M and Garfield RE (1994) An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. *Am J Obstet Gynecol* 170:175–185.

Send reprint requests to: Carl P. Weiner, M.D., Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Maryland School of Medicine, Bressler Research Bldg., Room 11-033, 655 W. Baltimore St., Baltimore, MD 21201. E-mail: cweiner@umm.edu